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EXHIBIT A

Vol. 33, No 5, August 1994 BIOCHEMISTRY *and* MOLECULAR BIOLOGY INTERNATIONAL
Pages 969–979

EARLY LYMPHOCYTE ACTIVATION EVENTS ARE INHIBITED BY ANTIPROLIFERATIVE SYNTHETIC PEPTIDE 2438, A FRAGMENT OF HUMAN INTERFERON ALPHA-2, AND IMMUNOSUPPRESSANT CYCLOSPORINE A.

Kirill V. Freze[*], Alla V. Danilkovich[*], Alexey I. Kharitonenkov[@], Alexander F. Shevalier[&], Alexander M. Surin[*], Alla B. Khodorova[*], Eugene I. Astashkin[*], Tamara V. Bulargina[@].

[*] - Department of Cell Physiology and Immunology, Biological Faculty, Moscow State University, Moscow, Russia;
[@] - Department of Bioorganic Chemistry, Biological Faculty, Moscow State University, Moscow, Russia;
[*] - Institute of Biotechnology, Moscow, Russia;
[&] - Institute of Bioorganic Chemistry, Moscow, Russia.

Received April 15, 1994
Received after revision, June 5, 1994

Summary.

A synthetic peptide (designated 2438) corresponding to the human interferon alpha-2 amino acid sequence 124-138 inhibits proliferation of T-lymphocytes in vitro. Time-course experiments suggest that peptide 2438 affects early stages of lymphocyte activation. Molecular mechanisms of peptide 2438 action were studied. By western-blotting with monoclonal antibodies against phosphotyrosine peptide 2438 was shown to decrease the phosphotyrosine content of an endogenous protein substrate (M.M.= 36 kDa) in human lymphocytes activated with concanavalin A (ConA). Similar effect on tyrosine-specific phosphorylation in mitogen-stimulated lymphocytes was observed with the native interferon or Cyclosporine A (CsA). Calcium fluxes induced by ConA in human lymphocytes were measured using a fluorescent calcium chelator Fura-2. In contrast to CsA, peptide 2438 did not affect the ConA-induced calcium influx in lymphocytes.

Introduction.

The main feature of immunosuppressive agents is their ability to inhibit the transition of resting lymphocytes to proliferation which prevents the clonal expansion. This kind of inhibition is usually accompanied by a decreased expression of genes coding for cytokines and their receptors. Earlier we reported that the

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The abbreviations used are: ConA, Concanavalin A; CsA, Cyclosporine A; IFN, interferon ; PBS, phosphate buffered saline PBMC, peripheral blood mononuclear cells; pp36, phosphoprotein with appropriate molecular mass 36 kD; P-Tyr, phosphotyrosine; $[Ca^{2+}]_i$, concentration of cytosolic free calcium.

Corresponding author: Kirill V. Freze, Department of Cell Physiology and Immunology, Biological Faculty, Moscow State University, 119899, Moscow, Russia.

1039–9712/94/050969–11$05.00/0

synthetic peptide 2438 from the carboxy terminus of human interferon alpha-2 inhibited mitogen-stimulated proliferation of human T-cells [1] and downregulated the activation-dependent increase in phosphotyrosine content of a phosphoprotein with a molecular mass of 36 kDa [2]. In contrast to the original molecule of interferon alpha-2, peptide 2438 neither possesses antiviral activity [3] nor affects lymphocyte viability [1]. Molecular mechanisms of the peptide 2438 action remain unclear. We suppose its antiproliferative activity may be coupled with the inhibition of tyrosine phosphorylation. In this study we made an attempt to elucidate the direct effects of antiproliferative agents, CsA and peptide 2438, on the processes stimulated in lymphocytes by a polyclonal mitogen, and to compare the peptide 2438 action with that of CsA. To this end, we determined the tyrosine content in phosphoproteins extracted from peripheral blood mononuclear cells treated with ConA and/or antiproliferative agents, synthetic peptide 2438 and CsA. We also examined the influence of peptide 2438 and CsA on ConA-induced calcium fluxes. In order to investigate effects of peptide 2438 and CsA on early stages of lymphocyte activation, inhibition of human T-cell proliferation was measured in time-course experiments.

Materials and Methods.

All media and media supplements for cell cultures were obtained from Sigma. Recombinant human IFN (Reaferon) was a gift of Dr. G.Chipens (Institute of Organic Chemistry , Riga, Latvia). CsA was from Sandoz (Sandimmune). Stock solution of CsA (1 mg/ml) was made in dimethyl sulphoxide. Fluorescent calcium chelator Fura-2 (Fura-2/AM) was from "Calbiochem" (Switzerland). Concanavalin A was from Pharmacia, Sweden. Monoclonal antibodies against P-Tyr were described earlier [4]. Radioactive ^{3}H-thymidine was purchased from Amersham. Other chemicals were of reagent grade.

Separation of cells.

Human blood was obtained from healthy volunteers. Mononuclear cells were isolated by gradient centrifugation according to Boyum [5].

Lymphocyte proliferation.

Cells were placed into 96-well tissue culture plates ($1x10^5$ cell/200 µl/well). Peptide 2438 (2 µg/ml) and CsA (0.1 µg/ml) were added to cells either 15 min before, or simultaneously, or else at

different periods of time after the addition of ConA. All experiments were done in triplicates. Cells were maintained in an atmosphere of 5% CO_2, 95% relative humidity, at 37°C for 72 hours. ^{3}H-thymidine (0.5 µCi/well) was added 15 hrs before termination of cultivation. The cells were harvested onto glass fiber filters and the radioactivity uptake was determined on an LKB-Wallac beta scintillation counter. Inhibition of proliferation was expressed in per cents according to the equation:

$$\text{Per cent of inhibition} = \frac{\text{experiment (cpm)}}{\text{control (cpm)}} \times 100,$$

where "experiment" stands for an average radioactivity value in the presence of antiproliferative agents, whereas "control" is the average radioactivity in their absence. Standard deviation was less that 10% in all experiments. The total number of experiments was 8.

Detection of phosphotyrosine in cellular phosphoproteins.

Freshly separated cells was washed in PBS and distributed into Eppendorf tubes in aliquotes of 10^6 cells per tube. The content of the tubes was resuspended in PBS, and allowed to equilibrate for 30 min at 37° C. The reaction was started by addition of peptide 2438 (2 µg/ml), CsA (1 µg/ml), IFN (1000 U) and/or ConA (2 µg/ml) to a final volume of 100 µl. Non-treated cells served as a control. Incubation time varied from 1 to 15 min. After the reaction was terminated the cells were centrifuged, the precipitates resuspended in 100 µl of sample buffer for electrophoresis (0.2 % SDS, 10% glycerol, 50 mM dithiothreitol, 62 mM Tris-HCl, pH 6.8), and immediately heated in boiling water for 5 min. Samples (25 µl) were applied to 10% polyacrylamide gels and electrophoresed under denaturing conditions according to Laemmli [6]. Separated proteins were blotted onto nitrocellulose filter using a semi-dry transfer system [7]. To prevent nonspecific binding of antibodies to nitrocellulose, blots were saturated by preincubation in blocking solution (1 % bovine serum albumin in 0.014 M NaCl, 0.005 M Na phosphate, pH=7.4, containing 0.05% of nonionic detergent Tween-20) for 1 hr. The preincubated blots were stained with monoclonal antibodies against P-Tyr, followed by rabbit antimouse antibodies, conjugated with horseradish peroxidase [4]. All dilutions of the antibodies were made in the blocking solution. The color reaction was developed by a peroxidase substrate, 1-chloro-4-naphtol. The gel lanes containing stained bands of polypeptides were scanned on an Ultroscan laser densitometer, and the optical density values were compared. The experiments were repeated 7 times.

Measurement of Ca^{2+} cytoplasmic concentrations in PBMC.

This method was earlier detailed by Grynkievicz [8] using the calcium chelator Fura-2. In brief, immediately after the separation PBMC suspensions were stained by incubating with 1 µM Fura-2/AM (acetoxymethyl ether of Fura-2) for 1 hr in Hank's solution without phenol red, buffered with 10 mM HEPES (pH 7.35). All experiments were run in the same media as above. After incubation the cells were washed twice and resuspended at a

concentration of $1x10^6$ cells/ml. All measurements of $[Ca^{2+}]_i$ were carried out at 37^0 C in quartz cuvettes on an F-4000 Hitachi spectrofluorimeter under stirring, the excitation and emission wavelengths being 336 and 510 nm, respectively. Graphic representation of $[Ca^{2+}]_i$ was made according to the equation: $[Ca^{2+}]_i = 224 \times (R_{observed}-R_{min})/(R_{max} - R_{observed})$ [8]. The number of experiments totalled six.

Results.

ConA induced proliferation.

Fig. 1 demonstrates the results of proliferation experiments. The maximum inhibition of proliferation was achieved when peptide 2438 or CsA were added 15 min before the addition of ConA, and the inhibition was weaker once the both agents were added simultaneously with the mitogen. Meanwhile, the antiproliferative effect of peptide 2438 was considerably lower when added after ConA, and completely disappeared in the case the cells were treated by ConA longer than for 60 min. Similar results were obtained for cells treated with IFN. In contrast, CsA inhibited

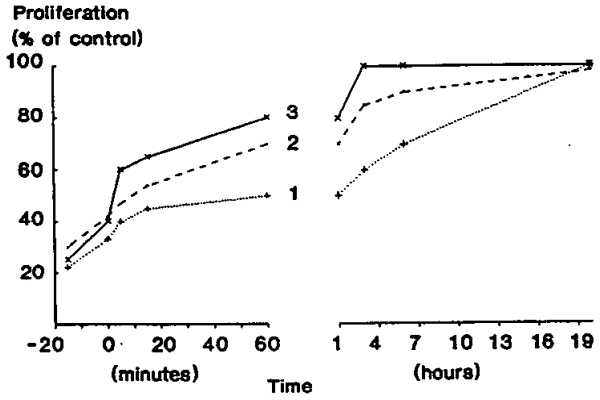


Figure 1.

Human PBMC proliferation in response to ConA in the presence of CsA (1), IFN alpha-2 (2), and synthetic peptide 2438 (3). Drugs were added to PBMC cultures at different time periods before and after the addition of ConA. X-axis is the time of drugs addition, where zero point corresponds to simultaneous addition of ConA and inhibitors of proliferation. Y-axis is proliferation in % in respect to control, where control is proliferation in the absence of drugs.

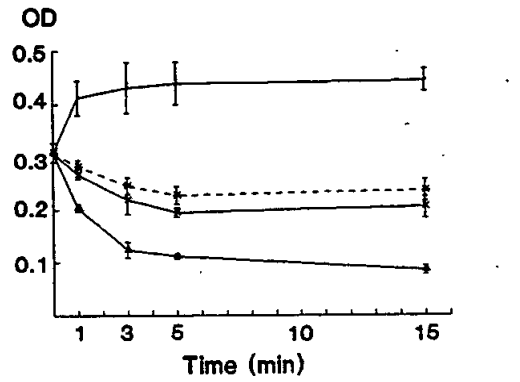


Figure 2.

Phosphotyrosine content of a 36-kDa polypeptide. Y-axis is the optical density values of stained bands ± CEM. X-axis is the incubation time. Cells were incubated with (1) - ConA; (2) ConA + peptide 2438; (3) - ConA + IFN; (4) - ConA + CsA.

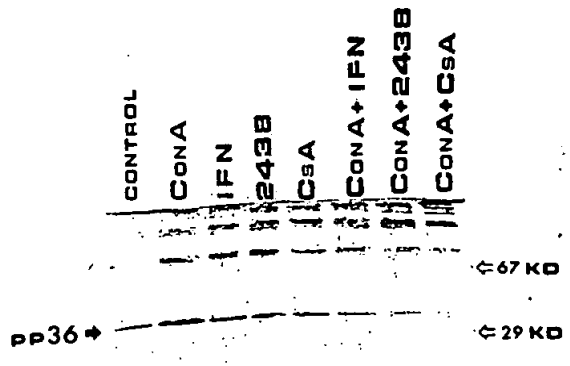
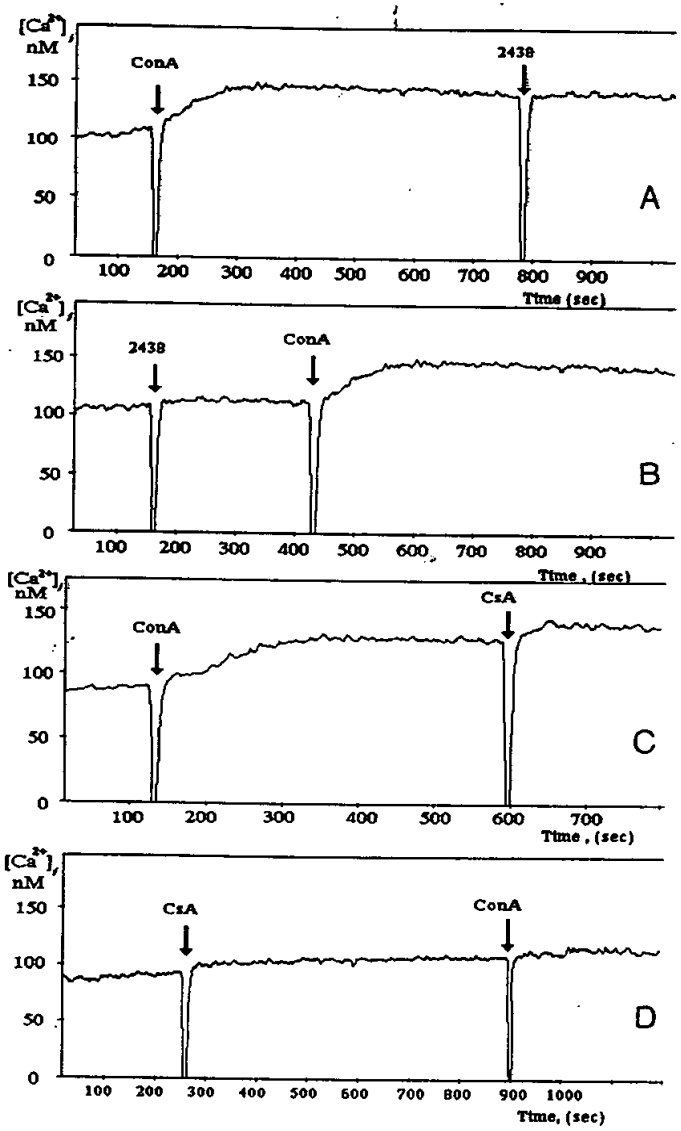


Figure 3.

Western blot of phosphoproteins from human PBMC incubated for 15 min with and without ConA, CsA, IFN and peptide 2438. Non-treated cells serve as a control.



Figures 4 A, B, C, D.

Concentration of cytosolic free calcium in PBMC under treatment with ConA, CsA and peptide 2438. X-axis is the time in minutes. The time and sequence of reagents added are indicated by arrows. Y-axis is Ca^{2+} concentration in nanomoles.

proliferation when added long after ConA, and this effect was significant even after 6 hrs of cell cultivation in the presence of the latter (data not shown).

Protein tyrosine phosphorylation.

Fig. 2 shows time-dependent changes in phosphotyrosine content of a 36-kDa phosphoprotein extracted from PBMC after the cells treatment with ConA as well as effects of peptide 2438 and CsA on tyrosine phosphorylation. The P-Tyr content in cells treated with antiproliferative agents alone remained unchanged (at a control level) (Fig. 3). ConA rapidly induced phosphorylation of a 36-kDa polypeptide (pp36) with the maximum P-Tyr level reached in 3 min (Fig 2). Simultaneous addition of ConA along with peptide 2438, IFN or CsA to the cell culture markedly decreased the P-Tyr levels of pp36 to values below the control (Fig. 3). Thus, antiproliferative agents induced a decrease in the P-Tyr content only in the ConA-treated cells.

Cytosolic Ca^{2+} concentration.

The concentration of cytosolic free calcium in the cytoplasm of non-activated cells was about 100 nM. Addition of the mitogen to the cells resulted in $[Ca^{2+}]_i$ increase, which reached its maximum at 140 nM, whereupon plateaued (Fig 4 A,B). In contrast, $[Ca^{2+}]_i$ was almost independent of peptide 2438, no matter whether the peptide was added before or after ConA (Fig 4 A, 5 A). Moreover, peptide 2438 did not prevent the mitogen-induced $[Ca^{2+}]_i$ elevation (Fig 5 A). CsA, as it is, increased $[Ca^{2+}]_i$ values at concentrations of 5-10 nM both when added before and after ConA (Fig 4 B, 5 B). In the meantime, CsA entirely blocked ConA-induced increments of $[Ca^{2+}]_i$ in all experiments (Fig 5 B). Thus, peptide 2438 and CsA differently influenced ConA stimulated calcium fluxes in PBMC.

Discussion.

In the preceding papers we studied the activities of various synthetic peptides derived from the carboxy terminal part of the human interferon α-2 molecule [1,2,9]. Only one of them, namely

2438, demonstrated antiproliferative activity against T-lymphocytes stimulated by ConA.

The antiproliferative effect of peptide 2438 was manifested only when added together with a mitogen, being undetectable once the peptide was added to activated cells after 24 hrs of cultivation [1]. Since the activated cells retained their peptide binding capacity [9], the resistance to the antiproliferative action of peptide 2438 cannot be explained by the loss of peptide receptors from the cell surface. It seems likely that peptide 2438 acts preferentially on non-stimulated cells and interferes with T-cell activation. The time-course experiments clearly show that inhibition of ConA-induced proliferation by peptide 2438 was observed provided the peptide was added before or immediately after the addition of ConA. Taken together, these findings strongly suggest that peptide 2438 affects preferentially early stages of lymphocyte activation. The time-course experiments also revealed that manifestation of IFN antiproliferative activity required conditions similar to those for peptide 2438, which may be indicative of at least partial resemblance of the antiproliferative action mechanisms for IFN and peptide 2438.

Our data with regard to Csa confirmed the result presented earlier by Lin that the inhibition of proliferation was severely diminished after 6 hrs of incubation with a polyclonal stimulator [10].

Recent observations demonstrated the key role of protein tyrosine phosphorylation in lymphocytes activation. T-cells stimulation by polyclonal activators leads to increase in tyrosine phosphorylation of endogenous substrates of protein tyrosine kinases, in particular the ζ chain of CD3 [11], and several polypeptides with molecular masses of 120, 80, 40 kDa [12]; 110, 90, 80 kDa [13]; 100, 84, 57, 38 kDa [14]; 94-100, 90, 64-75, 50-55, 36-40, 21 kDa [15].

Earlier we reported that peptide 2438 was able to lower P-Tyr content in the 36 kDa phosphoprotein (pp36), though measured 1 hr after the cells treatment with the peptide [2]. In this connection it was of interest to study the time course of ConA-induced tyrosine phosphorylation in the presence of peptide 2438, IFN and the widely used immunosuppressant CsA. The data obtained in this

study agree with the previous observations that pp36 phosphorylation is a marker of lymphocyte activation. The kinetic data presented here enable us to suppose that pp36 tyrosine phosphorylation is associated with early activation events.

It is generally accepted that antiproliferative activity of CsA also involves the inhibition of early stages of T-cell activation [10,16]. However, despite recent extensive investigations, the exact molecular mechanisms of CsA action are still unknown. Also, little is known on direct effects of CsA on a cascade of molecular events which follow the antigen-receptor binding.

The present investigation demonstrates that all the agents studied (CsA, IFN and peptide 2438) affect tyrosine phosphorylation in PBMC treated with the polyclonal mitogen ConA. In particular, they decreased the P-Tyr content in the endogenous substrate pp36, which can be a common constituent of the antiproliferative action of CsA and IFN. The latter were earlier reported to have common elements in their modes of antiproliferative action. Both agents caused a growth arrest in the G0/G1 phase of the cell cycle accompanied by a significant reduction in the levels of *c-myc* oncogene mRNA in a variety of sensitive hematopoietic cell types [17].

The action of the synthetic peptide 2438 on tyrosine phosphorylation in our experimental model was similar to that of both CsA and IFN. The role of the amino acid stretch 124- 138 in the antiproliferative action of IFN is still obscure. The most important question of whether or not this fragment of the interferon molecule possesses antiproliferative activity yet remains to be answered. The mechanism of the antiproliferative action of peptide 2438 may of course differ from that of IFN, but the features of their action (as well as for IFN and CsA) seem to have much in common.

We have obtained no direct evidence of the involvement of pp36 tyrosine phosphorylation in the cell growth regulation. It is only a good correlation between modulations of pp36 tyrosine phosphorylation and proliferation of cells that has been noted. Additional experiments would be essential for analysing the role of pp36 in the regulation of lymphocyte proliferation.

CsA is believed to inhibit calcium dependent pathways of the T-cell activation [10,18], but the data on direct CsA influence on cytoplasmic calcium concentration and mitogen-induced calcium influx are controversial, although this problem is a subject of many speculations. Some reports demonstrated that CsA did not inhibit $[Ca^{2+}]_i$ increase [19,20]. More recently, CsA has been shown to suppress the cytosolic $[Ca^{2+}]_i$ elevation [21]. However, close examination of the results of Metcalf [19] suggests an alternative interpretation of his data, the more so that they were obtained at times of CsA preincubation less than 30 min and the CsA concentration lower than 10 μg/ml. Other recent results confirm the previous observations that CsA inhibits increase in the activation-associated cytosolic $[Ca^{2+}]_i$ elevation [22]. Moreover, in our experiments, CsA itself slightly induces $[Ca^{2+}]_i$, which correlates with the data reported by Vereb for a human T-cell line HUT-78 [23].

Another finding is that, in contrast to CsA, peptide 2438 affected neither mitogen induced nor basal $[Ca^{2+}]_i$ levels in the cytoplasm of non-activated cells. According to the current view, cytosolic calcium rise is sufficient for IL-2 production [24]. It should be noted that peptide 2438 did not affect the production of IL-2 in ConA-activated human PBMC (A.V.Danilkovich, unpublished results). All these findings agree with an assumption that peptide 2438 does not affect Ca-dependent pathways of lymphocyte activation nor it interferes with IL-2 production. However, further studies are needed to finally elucidate the mechanisms of peptide 2438 action.

Acknowledgements:
The authors wish to express their appreciation to Dr. Boris O.Glotov (Institute of Molecular Genetics, Moscow) for valuable discussion and critical reviewing of the manuscript.

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CONTENTS

Volume 33, Number 4, July 1994

(Continued on inside back cover)

IU BMB A Publication of the International Union of Biochemistry and Molecular Biology

Biochemistry and Molecular Biology International

a rapid communication journal

Volume 33, Number 5, August 1994
Pages 817–1032 **ISSN 1039-9712**

ACADEMIC PRESS
Harcourt Brace & Company, Publishers
Sydney San Diego New York Berkeley
Boston London Tokyo Toronto